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(54) Title: MICELLE COMPOSITIONS CONTAINING PEGYLATED PHOSPHOLIPIDS AND A PHOTOSENSITIZER

(57) Abstract: The present invention relates to a non-toxic dipolar solvent for chromogenic substrate for detecting presence of lacZ gene and/or gene activity, which comprises a stabilizing amount of a solubilizing agent. The present invention also relates to a method for inducing lac operon in screening assay, comprising the step of contacting an agar plate with at least one essential oil in a concentration sufficient to induce the lac operon. The present invention further relates to a method for detecting the presence of bacteria, comprising the step of contacting an agar plate with at least one essential oil in a concentration sufficient to induce detection of the bacteria.

MICELLE COMPOSITIONS CONTAINING PEGYLATED PHOSPHOLIPIDS AND A PHOTOSENSITIZER

BACKGROUND OF THE INVENTION

(a) Field of the Invention

This invention relates a non-toxic solvent for preparing chromogenic substrate solution and uses thereof.

(b) Description of Prior Art

Many of the cloning and expression vectors in current use (e.g. the pUC series) carry a short segment of *E. coli* DNA that contains the regulatory sequences and the coding information for the first 146 amino acids of the ß-galactosidase gene (lacZ). Embedded in this coding region is a polycloning site that does not disrupt the reading frame but results in the harmless interpolation of a small number of amino acids into the amino-terminal fragment of ß-galactosidase. Vectors of this type are used in host cells that code for the carboxy-terminal portion of ß-galactosidase. Although neither the host-encoded not the plasmid-encoded fragments are themselves active, they can associate to form an enzymatically active protein. This type of complementation, in which deletion mutants of the operator-proximal segment of the lacZ gene are complemented by ß-galactosidase-negative mutants that have the operator-proximal region intact, is called α-complementation. The Lac+ bacteria that result from α-complementation are easily recognized because they form blue colonies in the presence of the chromogenic substrate 5-Bromo-4-chloro-3-indoxyl-ß-D-galactopyranoside (X-gal) (Horwitz et al. 1964. Substrates for cytochemical demonstration of enzyme activity. I. Some substituted 3-indoxyl-ß-D-galactopyranosides. J. Med. Chem. 7:574.). However, insertion of a fragment of foreign DNA into the polycloning site of the plasmid almost invariably results in the production of an amino-terminal fragment that is not capable of α-complementation. Bacteria carrying recombinant plasmids therefore form white colonies. The development of this simple color test has greatly simplified the identification of recombinants constructed in plasmid vectors of this type. It is easily possible to screen many thousands of colonies visually and to recognize

colonies that carry putative recombinant plasmids. The structure of these plasmids is then verified by restriction analysis of mini-preparations of plasmid DNA.

To a pre-made LB agar plate containing the appropriate antibiotics, a quantity of a stock solution of X-gal (20 mg/ml in dimethylformamide (DMF) or Dimethyl sulfoxide (DMSO)) and a quantity of a solution of isopropylthio- ß-D-galactoside (IPTG) is added. The stock solution of X-gal is usually prepared by dissolving X-gal in dimethylformamide or Dimethyl sulfoxide which is a toxic solvent presenting also the drawback of providing solutions that are not stable through time.

IPTG is an important addition to the blue-white screening. The vectors carrying a segment of DNA derived from the lac operon of *E. coli* that codes for the amino-terminal fragment of ß-galactosidase can be induced by isopropylthio-ß-D-galactoside (IPTG). Bacteria exposed to the gratuitous inducer IPTG synthesize both fragments of the enzyme and form blue colonies when plated on media containing the chromogenic substrate 5-Bromo-4-chloro-3-indoxyl-ß-D-galactopyranoside (X-gal).

It would be highly desirable to be provided with new solvents that are non toxic for preparing chromogenic substrate solutions used in screening assays, these solvents providing an extended stability of the chromogenic substrate solution.

SUMMARY OF THE INVENTION

In accordance with the present invention there is provided a non-toxic dipolar solvent for chromogenic substrate for detecting presence of lacZ gene and/or lacZ gene activity, which comprises a stabilizing amount of a solubilizing agent.

The solvent in accordance with a preferred embodiment of the present invention, wherein the solvent is a microemulsion.

The solvent in accordance with a preferred embodiment of the present invention, wherein the solubilizing agent is at least one selected from the group consisting of 1-Methylpyrrolidone (NMP), N'-dimethyl propylene urea (DMPU), Propylene carbonate (PC) and essential oil.

The solvent in accordance with a preferred embodiment of the present invention, wherein the essential oil is present in an effective solubilizing concentration for dissolving the chromogenic substrate.

The solvent in accordance with a preferred embodiment of the present invention, wherein the essential oil is selected from the group consisting of Abies alba, Aniba roseodora, Cedrus atlantica, Citrus aurantifolia, Citrus aurantium, Citrus bergamia, Citrus limon, Citrus paradisi, Citrus reticulata, Citrus sinensis, Cupressus sempervirens, Juniperus communis, Juniperus virginiana, Picea mariana, Pinus sylvestris, Ravensara aromatica, Rosmarinus officinalis, citrus extracts, pine terpenoids, conifers extracts, limonene oil and linseed oil.

In accordance with the present invention, there is provided a composition for detecting the presence of lacZ gene and/or lacZ gene activity comprising the solvent of the present invention and an effective amount of chromogenic substrate.

In accordance with the present invention, there is provided a method for inducing lac operon in screening assay, comprising the step of contacting an agar plate with at least one essential oil in a concentration sufficient to induce the lac operon.

The method in accordance with a preferred embodiment of the present invention, the lac operon being induced in one selected from the group consisting of *E. Coli*, Bacillus subtilis, phage, or *in situ* tissues.

In accordance with the present invention, there is provided a method for detecting the presence of bacteria, comprising the step of contacting an agar plate with at least one essential oil in a concentration sufficient to induce detection of the bacteria.

For the purpose of the present invention the following terms are defined below.

The term "chromogenic substrate" is intended to mean a substrate that produce a color when contacted with an appropriate reagent. The chromogenic substrate can be one of, but not limited to, X-Gal and IPTG.

All references herein are hereby incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates a bacterial culture exhibiting a strong blue color indicative of lac operon induction without the presence of IPTG when X-gal dissolved in essential oils;

Fig. 2 illustrates the results of plating a ligation/transformation onto LB plates containing X-gal dissolved in dimethylformamide (DMF);

Fig. 3 illustrates the results of plating a ligation/transformation onto LB plates containing X-gal dissolved in NMP and methanol;

Fig. 4 illustrates the results of plating a ligation/transformation onto LB plates containing X-gal dissolved in NMP, sea pine turpentine and methanol; and

Fig. 5 illustrates the results of plating a ligation/transformation onto LB plates containing X-gal solution aged of 16 months;

Fig. 6 illustrates the aging of LB plates at 4°C, room temperature, 37°C and 65°C (in the clockwise direction); and

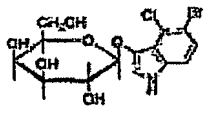
Figs. 7A-7C illustrate LB plates after 2 weeks at various temperatures.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there is provided non-toxic solvents for dissolving and stabilizing enzyme substrate used in screening assays.

One enzyme substrate widely used is X-gal, which is a dipolar molecule having the formula I:

5-Bromo-4-chloro-3-indoxyl-beta-D-galactopyranoside



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The X-gal solutions prepared with a non-toxic dipolar solvent of the present invention, for example 1-Methylpyrrolidone (NMP), N'-dimethyl propylene urea (DMPU), Propylene carbonate (PC), essential oils or a

combination of these, are very stables. In solution at 4°C, the X-gal will keep its activity for more than 6 months. If used to be poured in agar plates containing the proper antibiotic, these plates will remain active and usable for at least 3 months.

As an example of its non-toxicity, NMP is known as rapidly absorbed and eliminated. It is currently used intravenously in horses as a preanaesthetic. It is also used as an excipient in topical pharmaceutical formulations in human medicine and in cosmetics.

Essential oil mixes can be used to create a dipolar environment allowing X-gal dissolution. For instance, a combination of citrus extracts, pine terpenoids, limonene and linseed oil was shown to create an environment allowing the proper dissolution of X-gal.

Non-toxic micro emulsions can also be used to dissolve X-gal. A micro emulsion is a thermodynamically stable dispersion of one liquid phase into another, stabilized by an interfacial film of surfactant. This dispersion may be either oil-in-water or water-in-oil. Micro emulsions are typically clear solutions, as the droplet diameter is approximately 100 nanometers or less. The interfacial tension between the two phases is extremely low. Emulsions are in contrast unstable, the suspended droplets will eventually agglomerate and the dispersed phase will phase separate. Emulsion droplet sizes are much larger, typically one micron or more, resulting in a cloudy or milky dispersion. The nature of an emulsion may depend on the mixing of the ingredients and the amount of energy put into the mixing process.

A combination of essential oil was extracted with chloroform in order to remove part of the oil phase, and create a semi-precipitated emulsion. For example, 20ml of citrus extract, pine terpenoids, limonene oils and linseed oil was mixed with 20ml of Chloroform by vigorously shaking in a 50ml falcon tube. The resulting mix was let stand for approximately one day, and then the upper phase was transferred to a 15ml falcon tube. The obtained phase is a milky emulsion. It dissolves X-gal very well. If we let stand the milky phase for approximately one week, it will show the development of 5 distinct phases. Isolating each phases, it was interesting to observe that only phase # 2, the milky sub-phase, then

still an emulsion, can be used as an active solution to dissolve X-gal. The active phase is yellow milky cloudy in appearance. Using 500 µl completely dissolve 0.01g of X-gal; however, it gives a very opaque and viscous solution in appearance.

Micro emulsions are proper to create dipolar environment allowing a complete dissolution of X-gal. A mixture of oil, egg yolk or lecithine and acetic acid was sufficient to dissolve X-gal and show blue bacteria when used for its function as spread on an agar surface. Linseed oil, which has an average composition of different fatty acids (C16:0 palmitic acid 4-9%, C18:0 stearic acid 2-4%, C18:1 oleic acid 14-39%, C18:2 limoleic acid 7-19%, C18:3 limolenic acid 35-66%) is a known carrier for lipophilic molecules (as essential oils) and can act as a compound of the oily phase of the emulsion.

A micro emulsion is ideally made of a non-polar liquid mixed with a polar liquid in the presence of a surfactant or amphiphile, which is ideally a molecule carrying both polar and non-polar charges. In our situation, the surfactant, or amphiphilic molecule is X-gal. The role of the surfactant is to reduce the interfacial tension between two partially miscible or immiscible fluids below that obtained when no surfactant is present.

As shown in Table 1, variation in the composition of the micro-emulsion has an impact on the temperature flash point, which is a concern for transportation purposes.

Table 1

X-Gal with	Flash point (°C)
10% water / 90% NMP	> 93
5% water / 95% NMP	88
1% seapine / 10% water / 89% NMP	76
1% seapine / 99% NMP	73
10% seapine / 90% NMp	47.5

As well, IPTG is a usual and relatively essential addition to the cloning process. Omitting IPTG from the growth medium will decrease the expression level from plac, blue /white selection is usually not possible in the absence of IPTG. *E. coli* lac operon consists of a promoter, a transcriptional regulatory site called the operator (o), a CAP binding site (c), and three structural genes (lacZ, lacY and lacA) that are transcribed as a single polycistronic mRNA. Transcription of the lac operon is regulated by the lac repressor protein (lacI) which is encoded on a gene physically linked to the lac operon. lac operon inducers, such as IPTG, inactivate the lac repressor protein resulting in transcriptional de-repression of the lac operon. It is possible to artificially induce the lac operon using a nonmetabolizable allolactose analogue, isopropylthiogalactoside (IPTG), which binds to the lac repressor protein.

It is shown here that essential oils are replacing IPTG to induce the lac operon. It was found that compositions comprising essential oils not only dissolves X-gal, but also enhances the blue color without the need of IPTG. Among different tested essential oils, the most interesting alternative to IPTG is a small amount of the essential oil Sea Pine. Other essential oils extracted from spruce, pine or other conifers are also candidate to replace IPTG. Using such oil instead of IPTG represent different benefits, being non-toxic, all natural, easily biodegradable, low cost and most importantly, already as a liquid solution, ready to use.

Monoterpenes and sesquiterpenes were shown to be successful in dissolving X-gal and replacing IPTG in screening clones using the blue/white X-gal selection method.

In addition to X-gal , it is also possible to dissolve IPTG with the same solutions for cloning with bacterial systems needing that stimulation (see Table 2 for examples). These same solutions allow stable dissolution of Ampicillin, Tetracyclin and Chloramphenicol, providing an approach wherein a complete solution is available to the user. To dissolve Kanamycin, a solution with a base of micelles in aqueous solution is essential.

Table 2

Strain	(lacZ)M15	laclq	F'	Cam R	Kan R	Str R	Tet R	Comments
DH5-alpha	x							Invitrogen: (lacZYA)U169
DH5-alpha E	x							Invitrogen; (lacZYA)U169
DH5-alpha F'	x		x					Invitrogen; (lacZYA)U169
DH5-alpha T1R	x							Invitrogen; (lacZYA)U169
DH10B	x							Invitrogen
DH10Bac	x							Invitrogen; for producing recombinant baculovirus molecules
DH10B T1 R	x							Invitrogen
GeneHogs	x							Invitrogen; D10B derivative
INV-alpha F'	x		x					Invitrogen, (lacZYA)U169
JM83	x					x		ATCC 35607
Select 96	x							Promega
TB1	x					x		NEB; comes with pMAL system
TOP10	x							Invitrogen
TOP10/P3	x				x	x	x	Invitrogen; AMP R
ABLE C	x	x	x		x		x	Stratagene; lower plasmid copy number
ABLE K	x	x	x		x		x	stratagene; lower plasmid copy number
AD494								
BB4	x	x	x				x	=LE392.23
BMH 71-18 mutS	x	x	x				x	
DH5-alpha F' IQ	x	x	x		x			Invitrogen
DH-alpha FT	x	x	x				x	Invitrogen; (lacZYA)U169
DH11S	x	x	x					Invitrogen; mainly for ssDNA production with phagemid/m13
DH12S	x	x	x				x	Invitrogen
Electro Ten blue	x	x	x		x		x	Stratagene
ER1727	x	x	x			x	x	NEB
ER2267	x	x	x					NEB
ER2738	x	x	x					NEB
INV110	x	x				x	x	Invitrogen; to produce umethylated DNA
JM101	x	x	x					ATCC33876

Strain	(lacZ)M15	laclq	F'	Cam R	Kan R	Str R	Tet R	Comments
JM103	x	x	x			x		ATCC 39403
JM105	x	x	x			x		ATCC47016
JM107	x	x	x					ATCC47014
JM109	x	x	x					ATCC 53323; to maintain F' grow on M9 with 1 mM thiamine
JM110	x	x	x			x		ATCC 47013
NM522	x	x	x					ATCC 47000
NM527								
SCS110	x	x	x			x		Stratagene
Solo Pack gold	x	x	x	x			x	Stratagene
SOLR	x	x	x		x			Stratagene; excision assist strain
Stbl4	x	x	x				x	Invitrogen; to clone unstable DNA
SURE	x	x	x		x		x	Stratagene
SURE-2	x	x	x	x	x		x	Stratagene
TG1	x	x	x					
TKX1	x	x	x		x		x	Stratagene; for prot production
TOP10 F'	x	x	x			x	x	
W3110								
XL1-Blue	x	x	x				x	Stratagene
XL1-Blue MRF'	x	x	x				x	Stratagene
XL1-Blue MRF' Kan	x	x	x		x		x	Stratagene
XL2-Blue	x	x	x	x			x	Stratagene
XL2-Blue MRF'	x	x	x	x			x	Stratagene
XL10gold	x	x	x	x			x	Stratagene
XL10gold KanR	x	x	x		x		x	Stratagene
XL mutS Kan S	x	x	x				x	Stratagene
XL mutS Kan R	x	x	x		x		x	stratagene
XLOLR	x	x	x				x	Stratagene; excision assist strain
XPORT	x	x	x					
G1698		x						
C1724		x						
96 pack gold	x	x	x	x			x	
EC100	x							Epicentre; pIndigo
EC300	x							Epicentre; copy control BAC
CC300	x							Epicentre; copy control BAC
EPI100	?	?						Epicentre; fosmid
EPI300	?	?						Epicentre; fosmid
AG1								DH1 derivative

Strain	(lacZ)M15	laclq	F'	Cam R	Kan R	Str R	Tet R	Comments
BL21 (DE3)							x	for prot expression; some also Cam R or Kan R
BNN93								
C600								ATCC 23724
CJ236			x	x				
DB3.1								Invitrogen; for propagating vector with ccdB gene
DH1								ATCC 33849
DH5 alpha MCR								Invitrogen; (lacZYA)U169

In a preferred embodiment of the present invention, individual LB plates are prepared adding 100 µl of X-gal solution to the surface of a LB plate being at room temperature and spreading evenly across the surface. The plate is dried before use. X-gal containing LB + Amp plates are stable for up to 90 days when stored at 4°C.

Batches can be prepared by aseptically adding X-gal solution directly to melted LB agar (temperature 50°C). 100 µl of the solution should be used for every Petri dish. For example, for each 500 ml add 2000 µl of X-gal solution. Mix well (for 3 to 5 minutes) and pour as you normally would. Let cool. X-gal LB plates are stable for at least 3 months when stored at 4°C. X-gal can be added before or after the addition of selective antibiotics to the medium. For best mixing results, a magnetic stir bar should be added during the autoclaving process or a sterile magnetic stir bar could be ascetically added after autoclaving.

In a preferred embodiment of the present invention, X-gal solution is prepared as follow:

NMP or DMPU only

Add 7 ml of either NMP or DMPU to 1 gram of X-gal. Stir until dissolved and bring to 10 ml with NMP or DMPU (whichever is already used).

NMP or DMPU with essential oil

Add 3.5 ml of either NMP or DMPU to 1 gram of X-gal.Stir until dissolved and bring to 5 ml with NMP or DMPU (whichever is already used). Mix with 5 ml of essential oil until thoroughly mixed.

NMP/methanol solution

Add 7 ml of either NMP or DMPU to 1 gram of X-gal and stir until dissolved. Bring to 10 ml with NMP or DMPU (whichever is already used). Add 90 ml of methanol and mix thoroughly.

Example 1

Essential oils as solvent and IPTG replacement

Using 0.01 g X-gal in 500 µl of TURPENOID NATURAL® (comprising a combination of citrus extracts, pine terpenoids, limonene and linseed oil) provides excellent dissolution of X-gal and without affecting bacterial growth. It also provides a strong blue color without the need of IPTG, as shown in Fig. 1.

Example 2

Dissolution of X-gal

10 mg of X-gal powder were successfully dissolved in the solvents described in the Table 3 below.

Table 3

Mix	**T1-**
Citrus lemon/Citrus sinensis	700 µl
Pinus pinaster	300 µl
Mix	**T2-**
Cupressus sempervirens	50 µl
Pinene (Sigma)	50 µl
Campher oil	100 µl
Citrus sinensis	800 µl
Mix	**T3-**
NMP	100 µl
Methanol (only to increase total volume):	*900 µl*
% NMP :	*10%*
Mix	**T4-**
PC	200 µl
Methanol (only to increase total volume):	*400 µl*
% PC :	*33%*

Example 3

Dissolution in NPM and NMP/methanol

A 10X X-gal/NMP solution could be stored at –20°C without freezing. Over time, the solution will take a very pale yellow color that does not seem to darken over time. A 10X X-gal/NMP solution is easily diluted to 1X in anhydrous methanol or 95% ethanol. When diluted in methanol or ethanol, the solution will not freeze.

When the 1X X-gal/NMP/methanol is stored for 16 weeks at 4°C, there is no loss in X-gal activity as measured by applying 100 µl to a LB plate, streaking *E. coli* pUC19 transfectant, incubation at 37°C and examination for blue colonies after 16 hours.

Tables 4 and 5 are showing the results obtained by testing the activity of X-gal in solution with different solvent on a weekly basis. In Table 4, the tested solutions were stored at 4°C. At weekly intervals, 100 µl was applied to an LB+Amp plate and streaked with *E. coli* containing pUC19. The plate was incubated overnight at 37°C. The following morning the plate was examined for blue colonies. In Table 5, LB + Amp plates were prepared and stored at 4°C. At weekly intervals, a plate was removed and streaked with *E. coli* containing pUC19. The plate was incubated at 37°C overnight. The blueness of the colonies was then scored.

Table 4

Weekly Testing of X-gal/NMP/Methanol solution

Week	Test Results
0	+
1	+
2	+
3	+
4	+
5	+
6	+
7	+
8	+
9	+
10	+
11	+

Week	Test Results
12	+
13	+
14	+
15	+
16	+
17	+
18	+
19	+
20	+

Table 5

Weekly testing of X-gal/NMP or X-gal/NMP/oil

Date	NMP plate	Oil+NMP plate
0	+	+
1	+	+
2	+	+
3	+	+
4	+	+
5	+	+
6	+	+
7	+	+
8	+	+
9	+	+
10	+	+
11	+	+
12	+	+
13	+	+
14	+	+

Example 4

Dissolution with Essential Oils

It was shown that when X-gal was first dissolved in NMP, mixed one to one with essential oil, and then diluted in methanol to 10 mg/ml the mixture would ultimately yield colonies that are darker blue than when X-gal is made up in dimethylformamide.

This work was repeated and expanded to include other essential oils. The oils tested were: Natural Orange Terpene Solvent (Eco-House);

Blue Gum Eucalyptus Organic (Divine Essense); Atlas cedarwood (Pranarom); Sea Pine Turpentine (Pranarom) and Natural Turpenoid.

In this experiment, a 100 mg/ml solution of X-gal in NMP was prepared. A one to one mix with each essential oil was then made using this solution. The net X-gal concentration is now 50 mg/ml. This solution was then dilute 5X with 100% methanol such that the final concentration of X-gal is 10 mg/ml. One hundred microliters of this solution is then applied per LB plate.

As a control, 50 µl of a 20 mg/ml solution of X-gal dissolved in dimethylformamide was applied to one LB plate.

E. coli containing pUC19 was streaked onto each "X-gal spread" LB plate for isolated colonies. The plates were incubated at 37°C overnight. After incubation, the growth on each X-gal plate was scored for blueness. The ranking was as follows (least blue to most blue): A (least); B and X-gal/DMF; C, D and E (most blue).

The results showed that when either Atlas Cedarwood, Sea Pine Turpenine or Natural Turpenoid were mixed with X-gal/NMP/methanol there was an enhanced blueness of the *E. Coli* pUC containing bacteria.

During assays with Sea Pine Turpentine, the amount of Sea Pine Turpentine added to the X-gal/NMP solution was serially diluted one in two four times, mixed with methanol and then spread onto LB plates. The amount of X-gal added to each plate was the same. The plates were then streaked with *E coli* containing pUC19 and incubated overnight.

An examination of the plates showed that the bluest colonies were those obtained with the original amount of Sea Pine Turpentine gave the deepest blue color.

In the previous experiments, the final X-gal concentration was 10 mg/ml. The concentration of X-gal was reduced to 7.5, 5 and 2.5 mg/ml. Plates were prepared and streaked. The net result was that there was a significant drop in blue coloration when the X-gal was dropped from 7.5 to 2.5 mg/ml. In a preferred embodiment of the present invention, X-gal is in a concentration of 10 mg/ml.

Ligation assays were performed using lambda DNA digested with PstI and pUC19 digested with PstI and CIAP treated. The completed ligation was transformed into DH5α and plated onto LB plates containing:

(a) X-gal dissolved in Dimethylformamide

(b) X-gal dissolved in NMP and methanol

(c) X-gal dissolved in NMP, Sea Pine Turpentine and methanol

The plates were incubated overnight. The results for a, b and c are shown in Figs. 2, 3 and 4, respectively. For all three plating, there were both white and blue colonies. Moreover, it is shown that the blues colonies of Fig. 4 are of a more intense blue than the ones of Figs. 2 and 3.

It is possible to incorporate X-gal directly in molten LB agar. To test this with the X-gal solution of the present invention, 500 ml of LB agar was made, autoclaved and cooled to about 50°C. To this 2 ml of a 10 mg/ml X-gal/NMP/SeaPine/methanol solution was added and mixed. Upon the addition of the X-gal solution, there was a cloudy appearance throughout the agar as it mixed. Mixing for 3 to 4 minutes did not disperse the cloudiness. However, when the plates were poured, the cloudiness dispersed upon cooling and solidification. A slight surface cloudiness was noted several hours later when the plates were inverted for incubation overnight at room temperature. However, the next day the plates looked normal.

One plate was used to streak a white and a blue colony and incubated overnight at 37°C.

Two plates were left at 25°C. These two plates served as shipping simulators. The plates were tested after 5 days and were streaked with a blue and white colony and incubated overnight.

Aging studies have been set up for the liquid product both with and without Sea Pine Turpentine. The studies conducted with the X-gal/NMP/methanol solution shown that the product is stable for 17 weeks at 4°C with only a minor pale yellow color developing over time. This has being conducted at the same time with the X-gal/NMP/SeaPine/methanol product.

Example 5

Aging studies

X-gal solution using NMP and/or essential oil were shown to have an improve shelf-life. Table 6 is providing life span of X-gal solutions.

Table 6

Temperature	Time
4°C	16 months
Room temperature	4 months
37°C	1 month
65°C	1 month

Moreover, it had been found that XGal Petri dishes were still active when conserved at 4°C during as long as 12 months.

Method and results

Longest-term recipe is 1st June of 2002 in amber bottles and stored at 4°C. The recipe is 10% NMP and 90% methanol. The solution was last test Sept 25 2003 and found to be functioning fine (see Fig. 5). Currently, only a pale yellow color and not crystals.

X-gal containing LB plates were prepared July 30 2002. Two sets of plates were poured and stored at 4°C only. The first set was X-gal dissolved in NMP then an equal volume of natural turpenoid extract was added followed by ethanol. The final X-gal concentration in the solution was 10 mg/ml. Ampicillin prepared in water was added separately to the molten agar before pouring.

Each week one plate was removed, streaked with pUC/DH5α and DH5α and incubated at 37°C overnight. During the course of the experiment, the streaked pUC/DH5α always turned blue and the DH5α did not grow. Up until July 30 2003, both Ampicillin and X-gal were functioning.

Tables 7-9 provide results from more aging studies performed with solutions in accordance with the present invention

Table 7

10% NMP

Main Ingredients	May 12	June 3	June 9	June 13	June 27	July 8	Aug 5	Aug 15	Sept 22
X-gal (ethanol) May 5, 2003	No crystals, clear solution , Ok	Clear, no crystals	Clear, no crystals	Yellow, no crystals	Yellow, no crystals ok	Yellow, no crystals ok	Yellow color, no crystals, ok	Slightly yellow, no crystals ok	Slightly yellow, no crystals ok
X-gal (methanol) May 1, 2003	No crystals, clear solution Ok	Clear, no crystals	Clear, no crystals ok	Clear, no crystals ok	Clear, no crystals ok	Clear, no crystals ok	Clear, no crystals ok	Clear, no crystals	Clear, no crystals
Ampicillin + X-gal May 6, 2003	No crystals, slight yellow color Ok ok	Yellow, No crystals, Ok ok	yellow color, no crystals	yellow color, no crystals Ok ok	yellow, no crystals Ok ok	Yellow, no crystals Ok ok	Yellow, no crystals, Ok ok	Yellow, no crystals Ok ok	Yellow, no crystals Ok ok
Amp in NMP + X-gal May 15, 2003	NA	Pale yellow Ok ok	Yellow, no crystals Ok, ok	Yellow, no crystals Ok, ok	Yellow, no crystals Ok, ok	Yellow, no crystals Ok ok	Yellow, no crystals Ok ok	Yellow, no crystals	Yellow, no crystals Ok Ok
Chloramphenicol IPTG (low) + X-gal May 5, 2003	No crystals, clear solution Ok, ok	Pale yellow, no crystals, Ok, ok	Pale yellow, no crystals Ok ok	Pale yellow, no crystals Ok ok	Pale yellow, no crystals Ok ok	Pale yellow, no crystals Ok ok	Pale yellow, no crystals Ok ok	Pale yellow, no crystals	Pale yellow, no crystals Ok Ok
Chloramphenicol IPTG (high) + X-gal May 5, 2003	No crystals, clear solution Ok, ok	Pale yellow, no crystals, Ok, ok	Pale yellow no crystals Ok ok	Pale yellow, no crystals Ok ok	yellow color, no crystals Ok ok	Pale yellow, no crystals, ok, ok	Pale yellow, no crystals, ok ok	Pale yellow ,no crystals	Pale yellow ,no crystals Ok Ok
Tetracycline* + X-gal (April 4, 2003)	Yellow, no crystals, No growth of DH5α	Yellow, clear, no crystals, No growth of DH5α	Darker yellow, no crystals	Darker yellow, no crystals Ok ok	Darker yellow color, no crystals ok ok	Dark yellow, no crystals Ok ok	Dark yellow, no crystals Ok ok	Yellow, no crystals, ok ok	Yellow, no crystals, ok ok

Main Ingredients	May 12	June 3	June 9	June 13	June 27	July 8	Aug 5	Aug 15	Sept 22
Tetracycline (low) + X-gal (May 5 , 2003)	Yellow, no crystals	Yellow, no crystals	Yellow, no crystals	Yellow, no crystals Ok ok	Yellow, no crystals	Yellow, no crystals	Yellow, no crystals, Ok, ok	Yellow, no crystals	Yellow, no crystals Ok Ok
Tetracycline (high) + X-gal (May 5 , 2003)	Yellow, no crystals	Yellow, no crystals	Yellow, no crystals	Yellow, no crystals Ok, ok	Yellow, no crystals	Yellow, no crystals	Yellow, no crystals, Ok, ok	Yellow, no crystals	Yellow, no crystals Ok Ok

Table 8

AGING TEST #1

Formulations		Start date	Aging test (# weeks)	Result	Aging test (# weeks)
DMF + X-Gal	**RT**	*17-Sep-03*	1 week	Appearance of soln: clear, Intensity 4+	4 weeks
	-20	*mid-july-03*	~9 weeks	Appearance of soln: clear, Intensity 4+	~ 12 weeks
BT lot no. 024 (25% NMP)	**65C**	*17-Sep-03*	1 week	Appearance of soln: yellow, Intensity 4+	4 weeks
	37C	*17-Sep-03*	1 week	Appearance of soln: light yellow, Intensity 4+	4 weeks
	RT	*21-Aug-03*	5 weeks	Appearance of soln: clear, Intensity 4+	8 weeks
	4C	*5-Aug-03*	~7 weeeks	Appearance of soln: clear, Intensity 4+	~ 10 weeks
BT lot no. 004	**RT**	*21-Aug-03*	5 weeks	Appearance of soln: light yellow, Intensity 4+	8 weeks
	4C	*18-Feb-03*	~31 weeks	Appearance of soln: light yellow, Intensity 4+	~ 34 weeks
BT Amp, lot 019	**RT**	*21-Aug-03*	5 weeks	Appearance of soln: yellow, Intensity 3+	8 weeks
	4C	*16-May-03*	~ 18 weeks	Appearance of soln: yellow, Intensity 3+	~ 21 weeks

All were tested with DH5 + TrueBlue

BT lot no. 024 (25% NMP) has an intensity rated higher than others probably because the resulting evaporation made it more []

Table 9

AGING TEST NEW FORMULATIONS

Formulations		Start date	Week 2	Result	W4 (M1)
A + X-Gal / IPTG	65C	*28-Sep-03*	14-Oct-03	Appearance of soln: VERYyellow, Intensity 3+	28-Oct-03
	37C	*28-Sep-03*	14-Oct-03	Appearance of soln: light yellow, Intensity 4+	28-Oct-03
	RT	*28-Sep-03*			28-Oct-03
	4C	*28-Sep-03*	14-Oct-03	Appearance of soln: clear, Intensity 4+	28-Oct-03
	-20	*28-Sep-03*			28-Oct-03
D + X-Gal / IPTG	65C	*28-Sep-03*	14-Oct-03	Appearance of soln: VERYyellow, Intensity 3+	28-Oct-03
	37C	*28-Sep-03*	14-Oct-03	Appearance of soln: light yellow, Intensity 4+	28-Oct-03
	RT	*28-Sep-03*			
	4C	*28-Sep-03*	14-Oct-03	Appearance of soln: clear, Intensity 4+	
	-20	*28-Sep-03*			28-Oct-03
K + X-Gal / IPTG	65C	*28-Sep-03*	14-Oct-03	Appearance of soln: brown-orange, Intensity 3+	28-Oct-03
	37C	*28-Sep-03*	14-Oct-03	Appearance of soln: yellow, Intensity 4+	28-Oct-03
	RT	*28-Sep-03*			28-Oct-03
	4C	*28-Sep-03*	14-Oct-03	Appearance of soln: clear, Intensity 4+	28-Oct-03
	-20	*28-Sep-03*			28-Oct-03
BT PLUS	65C	*28-Sep-03*	14-Oct-03	Appearance of soln: greenish-brown, Intensity 3+	28-Oct-03
lot no. 028, 25% NMP	37C	*28-Sep-03*	14-Oct-03	Appearance of soln: light yellow, Intensity 4+	28-Oct-03
	RT	*28-Sep-03*			28-Oct-03
	4C	*26-Aug-03*	14-Oct-03	Appearance of soln: clear, Intensity 4+	28-Oct-03
	-20	*28-Sep-03*			28-Oct-03
DMF + X-Gal / IPTG	65C	*28-Sep-03*	14-Oct-03	Appearance of soln: orange, Intensity ---	
	37C	*28-Sep-03*	14-Oct-03	Appearance of soln: clear, Intensity 4+	28-Oct-03
	RT	*28-Sep-03*			28-Oct-03
	4C	*28-Sep-03*			28-Oct-03
	-20	*28-Sep-03*	14-Oct-03	Appearance of soln: clear, Intensity 4+	28-Oct-03
DMSO + X-Gal / IPTG	65C	*28-Sep-03*	14-Oct-03	Appearance of soln: greenish-brown, Intensity ---	
	37C	*28-Sep-03*	14-Oct-03	Appearance of soln: light yellow, Intensity 4+	28-Oct-03
	RT	*28-Sep-03*			28-Oct-03
	4C	*28-Sep-03*			28-Oct-03
	-20	*28-Sep-03*	14-Oct-03	Appearance of soln: clear, Intensity 4+	28-Oct-03

Test 14 oct was done with DH5 + TrueBlue

Test 28 oct was done with XL1 + PUC (also solutions are 65C were rought back to 1400uL)

In Fig. 6, dishes from the BT lot no. 024, as described in Table 8 are illustrated. The two top left dish was conserved at 4°C, the top right dish was conserved at room temperature, the bottom left dish was conserved at 37°C and the bottom right dish was conserved at 65°C.

In Fig. 7A, dishes from the lot A, as described in Table 9, are illustrated. The top dish was conserved two weeks at 4°C, the bottom left dish was conserved two weeks at 37°C and the bottom right dish was conserved two weeks at 65°C.

In Fig. 7B, dishes from the lot 028, as described in Table 9, are illustrated. The top dish was conserved two weeks at 4°C, the bottom left dish was conserved two weeks at 37°C and the bottom right dish was conserved two weeks at 65°C.

n Fig. 7C, dishes from the lot DMSO, as described in Table 9, are illustrated. The top dish was conserved two weeks at -20°C, the bottom left dish was conserved two weeks at 37°C and the bottom right dish was conserved two weeks at 65°C.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A non-toxic dipolar solvent for chromogenic substrate for detecting presence of lacZ gene and/or lacZ gene activity, which comprises a stabilizing amount of a solubilizing agent.

2. The solvent of claim 1, wherein said solvent is a microemulsion.

3. The solvent of claim 1, wherein said solubilizing agent is at least one selected from the group consisting of 1-Methylpyrrolidone (NMP), N'-dimethyl propylene urea (DMPU), Propylene carbonate (PC) and essential oil.

4. The solvent of claim 1, wherein said essential oil is present in an effective solubilizing concentration for dissolving said chromogenic substrate.

5. The solvent of any one of claims 3 and 4, wherein said essential oil is selected from the group consisting of Abies alba, Aniba roseodora, Cedrus atlantica, Citrus aurantifolia, Citrus aurantium, Citrus bergamia, Citrus limon, Citrus paradisi, Citrus reticulata, Citrus sinensis, Cupressus sempervirens, Juniperus communis, Juniperus virginiana, Picea mariana, Pinus sylvestris, Ravensara aromatica, Rosmarinus officinalis, citrus extracts, pine terpenoids, conifers extracts, limonene oil and linseed oil.

6. The solvent of claim 1, wherein said chromogenic substrate is selected from the group consisting of X-Gal and IPTG.

7. A composition for detecting the presence of lacZ gene comprising the solvent of any one of claims 1-6 and an effective amount of chromogenic substrate.

8. A method for inducing lac operon in screening assay, comprising the step of contacting an agar plate with at least one essential oil in a concentration sufficient to induce said lac operon.

9. The method of claim 8, said lac operon being induced in one selected from the group consisting of *E. Coli*, Bacillus subtilis, phage, or *in situ* tissues.

10. The method of claim 8, wherein said essential oil is selected from the group consisting of Abies alba, Aniba roseodora, Cedrus

atlantica, Citrus aurantifolia, Citrus aurantium, Citrus bergamia, Citrus limon, Citrus paradisi, Citrus reticulata, Citrus sinensis, Cupressus sempervirens, Juniperus communis, Juniperus virginiana, Picea mariana, Pinus sylvestris, Ravensara aromatica, Rosmarinus officinalis, citrus extracts, pine terpenoids, conifers extracts, limonene oil and linseed oil.

11. A method for detecting the presence of bacteria, comprising the step of contacting an agar plate with at least one essential oil in a concentration sufficient to induce detection of said bacteria.

12. The method of claim 11, wherein said essential oil is selected from the group consisting of Abies alba, Aniba roseodora, Cedrus atlantica, Citrus aurantifolia, Citrus aurantium, Citrus bergamia, Citrus limon, Citrus paradisi, Citrus reticulata, Citrus sinensis, Cupressus sempervirens, Juniperus communis, Juniperus virginiana, Picea mariana, Pinus sylvestris, Ravensara aromatica, Rosmarinus officinalis, citrus extracts, pine terpenoids, conifers extracts, limonene oil and linseed oil.

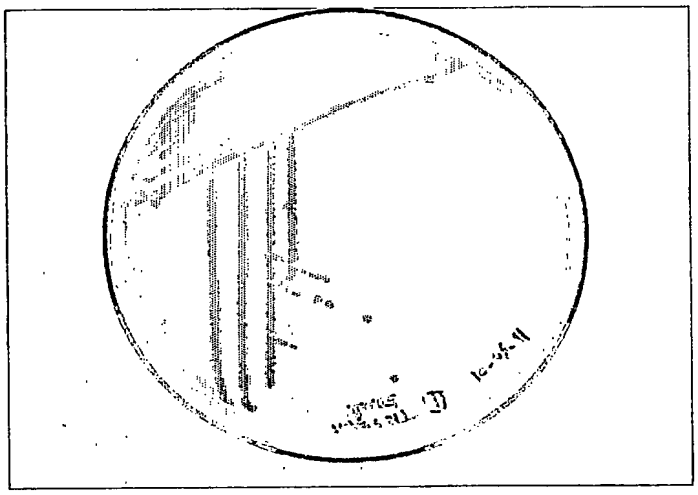

Fig. 1

Fig. 2

Fig. 3

Fig. 4

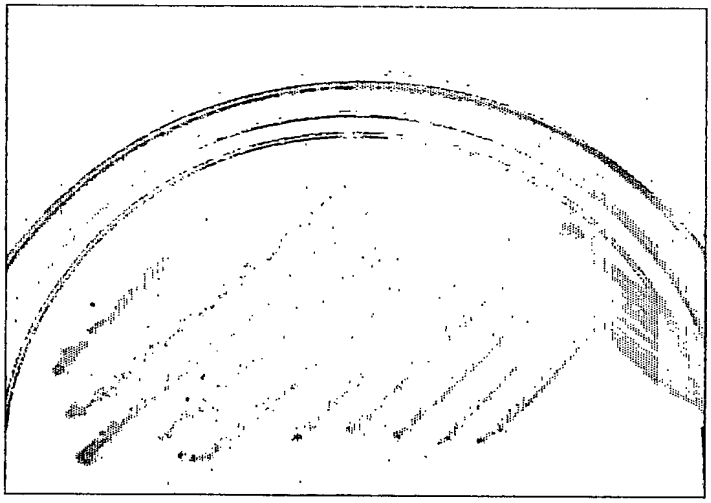

Fig. 5

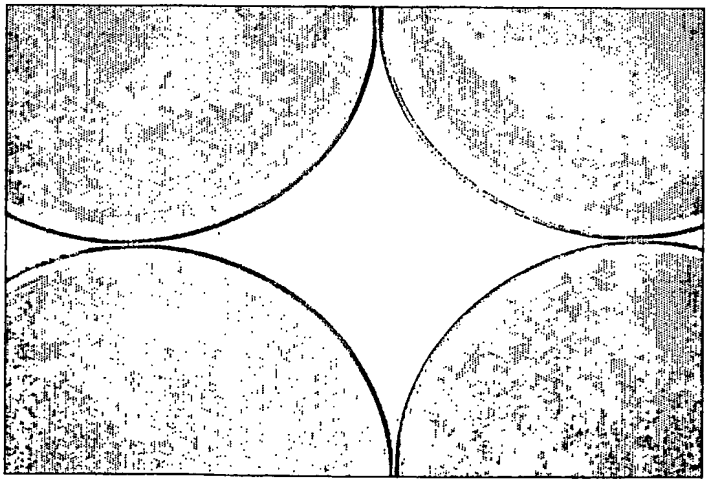

Fig. 6

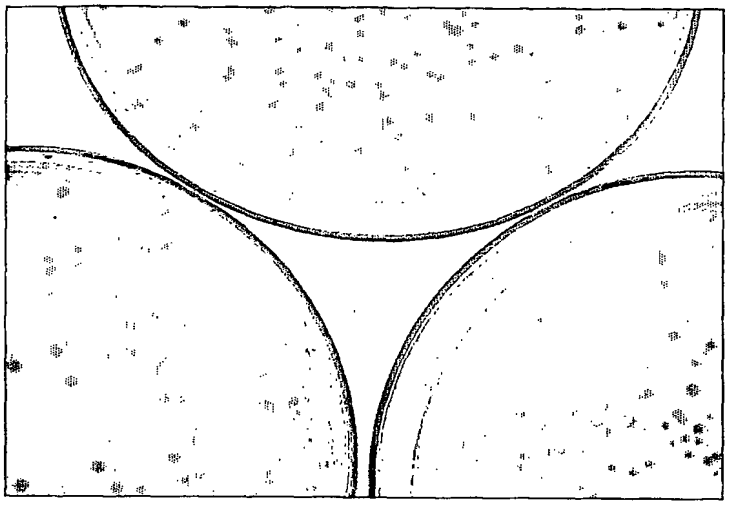

FIG. 7A

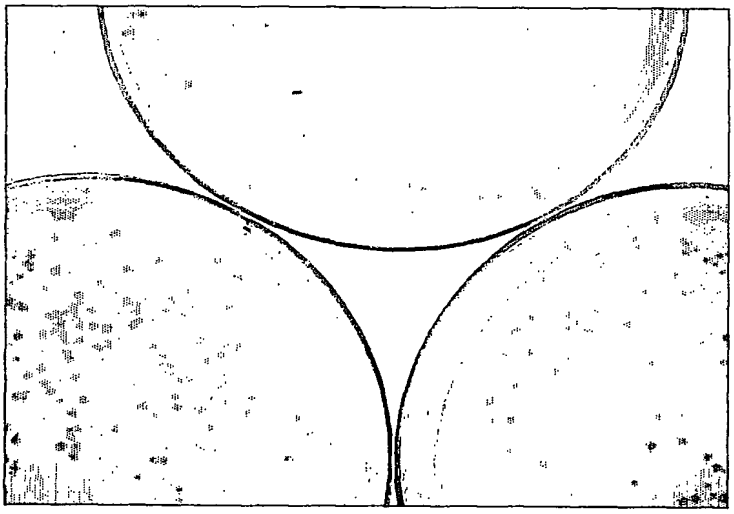

FIG. 7B

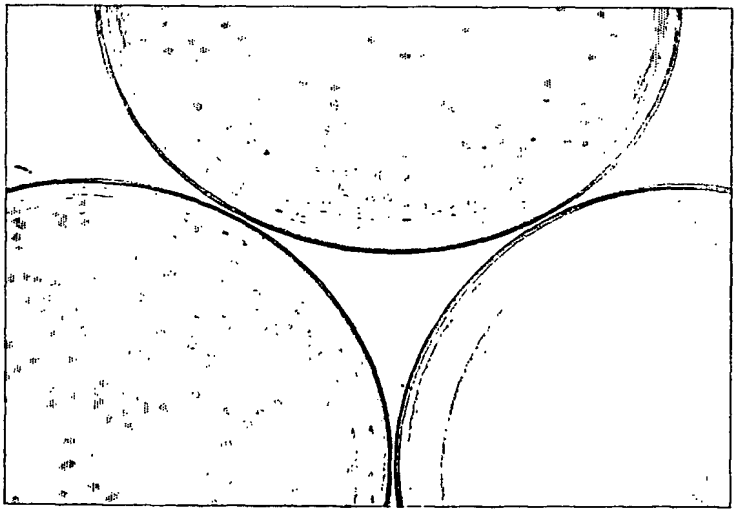

FIG. 7C

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 02/01690

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K9/107 A61K41/00 A61K31/409 A61K9/127

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
24 March 2004	16/04/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Giménez Miralles, J

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International Application No
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Published:
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8 July 2004

(15) Information about Correction:
see PCT Gazette No. 28/2004 of 8 July 2004, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: SOLVENT FOR CHROMOGENIC SUBSTRATE SOLUTION

(57) Abstract: The present invention relates to a non-toxic dipolar solvent for chromogenic substrate for detecting presence of lacZ gene and/or gene activity, which comprises a stabilizing amount of a solubilizing agent. The present invention also relates to a method for inducing lac operon in screening assay, comprising the step of contacting an agar plate with at least one essential oil in a concentration sufficient to induce the lac operon. The present invention further relates to a method for detecting the presence of bacteria, comprising the step of contacting an agar plate with at least one essential oil in a concentration sufficient to induce detection of the bacteria.

WO 2004/039354 A1

INTERNATIONAL SEARCH REPORT

Int[redacted]nal Application No
PCT/CA 03/01690

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98/50566 A (LEBEL SUZANNE ;SLILATY STEVE N (CA)) 12 November 1998 (1998-11-12) the whole document	8-12
A	EP 0 410 655 A (MYCOGEN CORP) 30 January 1991 (1991-01-30) the whole document	8-12
X	EP 0 354 027 A (ENVIROSOLV INC) 7 February 1990 (1990-02-07) claims 1,8	1-7
X	EP 0 950 403 A (ATRIX LAB INC) 20 October 1999 (1999-10-20) see claim 9 - a microemulsion; claim 10 - NMP and propylene carbonate; examples 1 and 2	1-3

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 May 2004	14/05/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Scott, J

Form PCT/ISA/210 (second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 317 042 A (NARAYANAN KOLAZI S) 31 May 1994 (1994-05-31) abstract	1-3
X	US 4 596 770 A (PARHAM MARC ET AL) 24 June 1986 (1986-06-24) abstract	1-3

INTERNATIONAL SEARCH REPORT

[redacted]national application No.
PCT/CA 03/01690

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-7 partially
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Continuation of Box I.2

Claims Nos.: 1-7 partially

Present claims 1 and 7 pertain to products - namely a solvent (claim 1) and a composition (claim 7) these claims cover an extremely large number of possible products. Support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the products claimed. Moreover there are many clarity problems with regard to the claims, which also make the scope covered by the claims even broader.

The terms which give particular clarity problems are :

non-toxic
chromogenic substrate
solubilizing agent
essential oil

Claim 1 seems to define a product (the solvent) in terms of the use to which it is put (i.e. for chromogenic substrate / for detecting the presence of lacZ)
"a stabilizing amount of a solubilizing agent" is a result to be achieved it is not clear whether an essential oil is an essential feature of the invention, and whether it is used in combination with NMP, DMPU and PC

Claim 1 can thus be seen as a non-toxic dipolar solvent comprising some solubilizing agent. Even looking at the more restrictive dependent claim 3, this can be seen as a non-toxic dipolar solvent containing NMP, DMPU, PC or essential oil, and mixtures thereof. A claim such as this would have so many novelty destroying documents that it is not meaningful to cite them all on the search report.

In the present case, the claims so lack support and are so unclear, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the the solvent of claim 1 and the composition of claim 7, in so far as the chromogenic substrate is present, and it is X-Gal or IPTG; and the solvent contains one of the group listed in claim 3 as the solubilizing agent; and the solvent is suitable for detecting the presence of lacZ or lacZ activity.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/CA 03/01690

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